2. Analysis of a protein extracted from wilted grass was not significantly different from that of fresh grass.

3. Analysis of a protein extracted from ensiled grass showed significant differences only in its content of serine and threonine, and these differences were small.

We wish to express our appreciation of the interest shown by Prof. E. L. Hirst, F.R.S. The work forms part of a programme of research on crop conservation sponsored by the Agricultural Research Council.

REFERENCES

Bailey, K. (1937). *Biochem. J.* **31**, 1396.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Crook, E. M. (1946). *Biochem. J.* **40**, 197.
Kemble, A. R. & Macpherson, H. T. (1954). *Biochem. J.* **56**, 548.
Lugg, J. W. H. (1949). *Advanc. Protein Chem.* **5**, 229.
Macpherson, H. T. (1952). *J. Sci. Fd Agric.* **3**, 362.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Yemm, E. W. & Folkes, B. F. (1953). *Biochem. J.* **55**, 700.

Liberation of Amino Acids in Perennial Rye Grass During Wilting

By A. R. KEMBLE and H. T. MACPHERSON
Chemistry Department, The King's Buildings, University of Edinburgh

(*Received* 15 *February* 1954)

Proteolysis during starvation of plant material gives rise to an increase in peptide, free amino acids and amides. The amides appear in amount greater than that contained in the protein and must incorporate nitrogen liberated by deamination of amino acids. Thus some of the amino acids released by proteolysis are metabolized further and do not appear in the calculated amount. For example, Wood & Cruikshank (1944) have shown cystine, glutamic acid, arginine, tyrosine and tryptophan to be oxidized in that order of rapidity.

No evidence is available concerning the remainder of the amino acids and the investigation has been extended to include the majority of the monoamino monocarboxylic acids.

EXPERIMENTAL

Analysis

Total nitrogen. The total nitrogen (TN) is that nitrogen in the plant determinable by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943). All results are expressed as a percentage of this TN.

Soluble nitrogen (SN). This is the fraction of the TN that is soluble in boiling water. Three successive extractions of 250 g. fresh material were made, each by 1 l. of boiling water.

Volatile base. The volatile base was determined by distilling a portion of the SN extract at pH 10·5 into standard acid.

Total amide. Asparagine and glutamine were hydrolysed by making a sample of the SN extract N with respect to H_2SO_4 and boiling under reflux for 3 hr. The increase in volatile base measures the NH_3 formed (Vickery, Pucher, Clark, Chibnall & Westall, 1935).

Glutamine. A sample of the SN extract was boiled under reflux at pH 6·5 for 2 hr. (Vickery *et al.* 1935). The increase in volatile base measures the NH_3 formed by the hydrolysis of glutamine. Some hydrolysis must have occurred during extraction but the resulting error cannot be great.

α-Carboxyl nitrogen. This was determined on a sample of the SN extract by the ninhydrin–CO_2 titrimetric procedure of Van Slyke, MacFadyen & Hamilton (1941).

Peptide. A sample of the SN extract (approximately 1 mg. N/ml.) was treated with 3 parts by vol. of ethanol. The resulting precipitate was removed by centrifuging and the N content of the precipitate determined. It is not claimed that this precipitate necessarily contains all the peptide present, but at least no α-carboxyl nitrogen is precipitated under these conditions and the determination is of value for comparison purposes.

Monoamino monocarboxylic acids. The ethanol was distilled from the supernatant solution obtained during the previous determination and a sample containing about 30 mg. N was subjected to ionophoresis. The neutral amino acids were estimated by the method of Kemble & Macpherson (1954*a*).

First wilting experiment

Young perennial rye grass (S. 24 hay strain; moisture content 88·3%; N, 4·56% of dry matter) was cut on 17 June 1953. The grass was 6–8 in. in height.

Six 250 g. samples were taken and the first was extracted immediately with boiling water. The remaining five samples were spread thinly on a laboratory bench and the SN was extracted after 1, 2, 3, 5 and 8 days respectively. The air temperature during the experiment was 14–18°.

Second wilting experiment

A mature sample of perennial rye grass (moisture content, 81·0%; N, 2·07% of dry matter) was cut on 28 October 1953. The grass (12–18 in.) was an aftermath and contained no flower heads.

Two samples (69·6 g. each) were taken. The first was allowed to wilt as before. The second was placed in a tall bottle and a slow stream of air saturated with water vapour was passed through the bottle continuously.

The SN was extracted from each after 3 days.

RESULTS

The results of the first wilting experiment are shown in Figs. 1–4. The fresh grass gave 0·5% of TN as peptide precipitable from the SN fraction. For wilted grass this figure rose to 2·5 and 3·3% after 1 and 8 days respectively. The values for the individual amino acids are compared with the values expected assuming uniform protein breakdown. These latter values are calculated from the expression

$$A_f + A_p\left(\frac{S_n - S_f}{100}\right),$$

where A_f is the free amino acid content of the fresh grass, A_p is the amino acid content of the protein and S_f and S_n are the soluble nitrogen fractions (% of

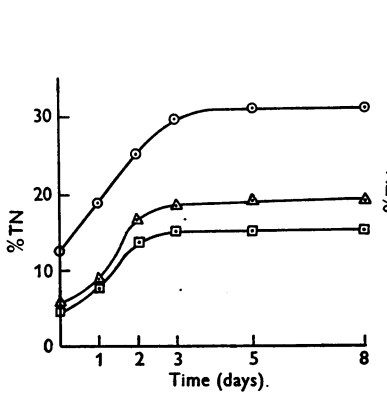

Fig. 1.

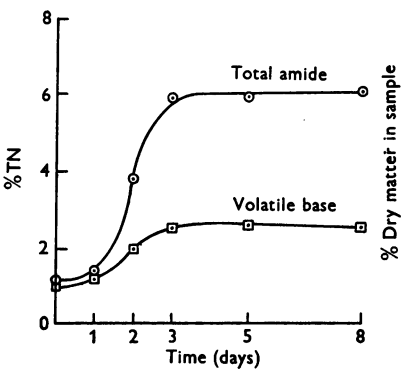

Fig. 2.

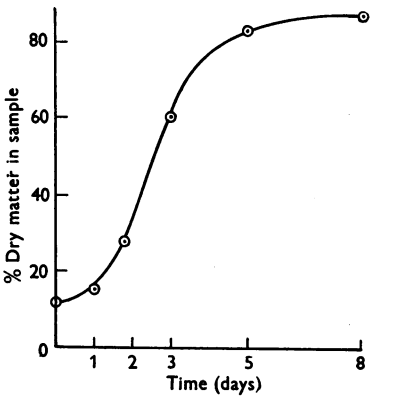

Fig. 3.

Figs. 1–3. Fig. 1. Soluble nitrogen (⊙), α-carboxyl N (⊡), and α-carboxyl N + volatile base + half amide N (△), formed in wilting grass (Expt. 1). Fig. 2. Formation of amide and volatile base in wilting grass. Fig. 3. Increase in dry-matter content of grass during wilting.

Table 1. *Proteolysis in two identical samples of cut grass kept 3 days under different conditions of humidity*

Original moisture content, 81·0%. TN 2·07% of dry wt.

	Wilting		Moist wilting*	
Original weight (g.)	69·6		69·6	
Weight after 3 days (g.)	19		68·6	
	N as % TN			
	Found	Expected	Found	Expected
Soluble N	30·6	—	38·6	—
Volatile base	0·5	—	1·5	—
Glutamine†	2·6	—	3·6	—
Asparagine†	4·8	—	10·4	—
α-Carboxyl N (less half amide N)	10·5	—	9·0	—
Glycine	0·09	1·15	0·15	1·68
Serine	0·81	1·01	1·24	1·34
Threonine	0·48	0·74	0·61	1·06
Hydroxyproline		Absent		
Alanine	0·41	1·26	0·26	1·73
Proline	3·91	0·85	0·53	1·13
Tyrosine	0·08	0·50	0·12	0·68
Valine	0·58	0·85	0·76	1·22
Methionine	0·08	0·24	0·03	0·38
Phenylalanine	0·43	0·58	0·26	0·93
Leucine	0·20	0·84	0·20	1·32
Isoleucine	0·36	0·53	0·36	0·84
Peptide N (precipitable in aq. ethanol, 75% v/v)	3·0	—	4·0	—

* See text for definition.

† The total amide N and asparagine/glutamine ratios are in excellent agreement with those recorded in a similar experiment (Macpherson, 1952).

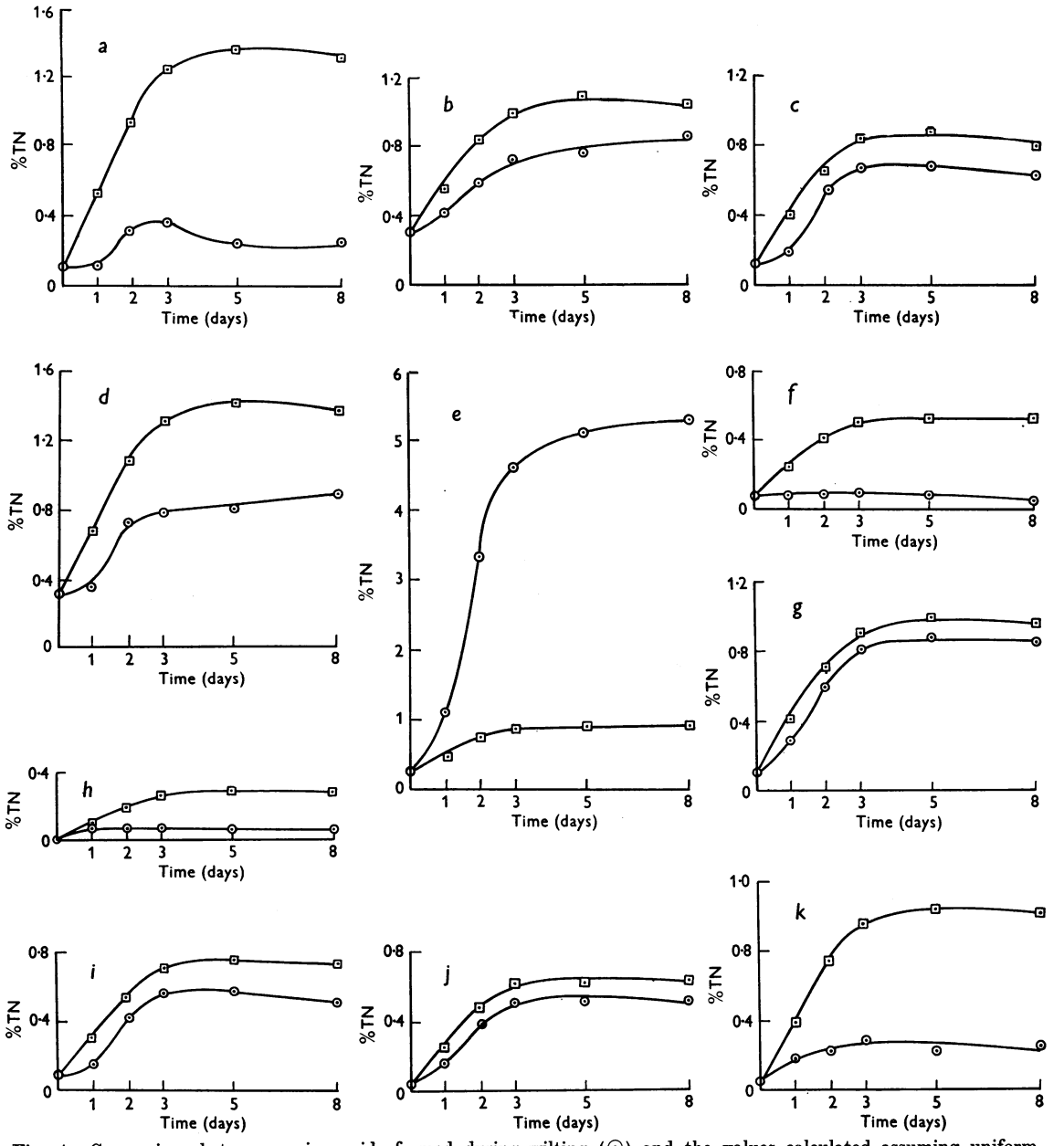

Fig. 4. Comparison between amino acids formed during wilting (⊙) and the values calculated assuming uniform breakdown of the protein (⊡). (*a*) Glycine; (*b*) serine; (*c*) threonine; (*d*) alanine; (*e*) proline; (*f*), tyrosine; (*g*), valine; (*h*), methionine; (*i*), phenylalanine; (*j*), isoleucine; (*k*), leucine.

TN) of the fresh grass and *n*th day's wilted grass respectively.

Values for the amino acid content of the protein are taken from Kemble & Macpherson (1954*b*) except for tyrosine (2·4 % of TN) and methionine (1·5 % of TN) (review by Lugg, 1949).

The results of the second wilting experiment are contained in Table 1.

DISCUSSION

Fig. 1 shows the overall course of proteolysis during the first wilting experiment. A considerable fraction of the soluble nitrogen is obviously not determined as amino acid, volatile base or amide. However, little or no increase in these undetermined substances took place during wilting as the small apparent

increase is accounted for by the increase in alcohol-precipitable peptide. The lag in α-amino acid liberation as compared with the increase in soluble nitrogen during the first 2 days is paralleled by the observed results for many of the individual amino acids (Fig. 4) and shows that the formation of free amino acid occurs via peptides rather than as a direct degradation of the protein molecule. This is confirmed by the sharp rise in the amount of soluble nitrogen precipitable by aqueous ethanol after the grass had wilted for 1 day.

The formation of amide was greatest during the second and third days after cutting (Fig. 2), as had been observed in detached barley leaves (Yemm, 1937), but cessation of nitrogen metabolism was virtually complete after 3 days, by which time the moisture content of the grass had fallen to approximately 40 % (Fig. 3).

Determinations of the individual amino acids in fresh grass (Fig. 4) were in agreement with the semiquantitative observations of Synge (1951). During the ensuing wilting all the amino acids, with the exception of proline, were found in amounts less than those calculated from protein degradation. The deficit was large for glycine, leucine and tyrosine and moderately large for alanine. This general pattern was also observed with a more mature grass (Table 1). It has been shown that the breakdown of protein during wilting is uniform (Kemble & Macpherson, 1954*b*) and so these deficiencies must be a measure of the rate at which the amino acids are metabolized after liberation.

Proline was the only amino acid which occurred in excess of expectation and this excess was very great. After 8 days the amount of free proline was 50 % in excess of that present in the original protein, even though only one-fifth of this had been degraded (Fig. 4*e*). Confirmation of this unexpected result was provided by the intensity of the distinctive yellow spot given by proline on a ninhydrin-sprayed chromatogram.

In a similar experiment carried out with mature grass, the pattern of glycine, leucine, tyrosine and alanine disappearance and of proline synthesis again emerged (Table 1). However, an identical sample of this grass which was not allowed to lose moisture during starvation ('moist wilting') did not synthesize proline. Thus some degree of desiccation would appear to be essential for proline formation.

There is little known concerning the synthesis of proline in higher plants but its formation in the rat can occur via glutamic acid (Sallach, Koeppe & Rose, 1951), synthesis of which, or at least its amide, can take place in starving plants. However, proline formation during wilting does not appear to be a simple matter of glutamine conversion, as the large difference in total amide between the wilting and moist wilting procedures is due almost entirely to the absence of asparagine (Table 1). Nevertheless, the accumulation of proline is comparable in magnitude with the amount of amide synthesized during moist wilting, especially if the comparison is made on a molecular basis rather than on nitrogen content, and it is possible that, under conditions of desiccation, proline synthesis undertakes a similar metabolic role to that normally provided by amide formation. In this respect it should be noted that there is no pronounced difference in the pattern of amino acid disappearance during the two wilting procedures (Table 1). Such a difference might have been expected if proline and amide synthesis were totally divorced from each other.

SUMMARY

1. The various groups of nitrogenous compounds formed by proteolysis in wilting grass have been estimated and it has been shown that all the nitrogen from the degraded protein reappears as α-amino acid, volatile base, amide or peptide.

2. Many of the free amino acids have been determined and the results compared with those expected from the extent of protein breakdown. Except for proline, all the amino acids were present in amounts smaller than calculated and these deficits varied greatly.

3. Proline was found in wilting grass in amounts greatly in excess of expectation but no synthesis occurred when the grass was not allowed to lose moisture during starvation. The possibility of a connexion between this synthesis and the known formation of amide during starvation is discussed.

We wish to express our appreciation of the interest shown by Prof. E. L. Hirst, F.R.S. The work forms part of a programme of research on crop conservation sponsored by the Agricultural Research Council.

REFERENCES

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.

Kemble, A. R. & Macpherson, H. T. (1954*a*). *Biochem. J.* **56**, 548.

Kemble, A. R. & Macpherson, H. T. (1954*b*). *Biochem. J.* **58**, 44.

Lugg, J. W. H. (1949). *Advanc. Protein Chem.* **5**, 229.

Macpherson, H. T. (1952). *J. Sci. Fd Agric.* **3**, 365.

Sallach, H. J., Koeppe, R. E. & Rose, W. C. (1951). *J. Amer. chem. Soc.* **73**, 4500.

Synge, R. L. M. (1951). *Biochem. J.* **49**, 624.

Van Slyke, D. D., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 671.

Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C. & Westall, R. G. (1935). *Biochem. J.* **29**, 2710.

Wood, J. G. & Cruikshank, D. H. (1944). *Aust. J. exp. Biol. med. Sci.* **22**, 111.

Yemm, E. W. (1937). *Proc. Roy. Soc.* B, **123**, 243.